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▣ MINI-REVIEW

Functional Genes and Proteins of *Clonorchis sinensis*

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Abstract: During the past several decades, researches on parasite genetics have progressed from biochemical and serodiagnostic studies to protein chemistry, molecular biology, and functional gene studies. Nowadays, bioinformatics, genomics, and proteomics approaches are being applied by Korean parasitology researchers. As for *Clonorchis sinensis*, investigations have been carried out to identify its functional genes using forward and reverse genetic approaches and to characterize the biochemical and biological properties of its gene products. The authors review the proteins of cloned genes, which include antigenic proteins, physiologic and metabolic enzymes, and the gene expression profile of *Clonorchis sinensis*.

Key words: *Clonorchis sinensis*, functional genes, chromosome, antioxidant enzyme, protease, energy metabolism, antigenic proteins

INTRODUCTION

Clonorchis sinensis-infections are the most prevalent form of parasitic infections in Korea today. Controlled mass chemotherapy has been deployed based on praziquantel in endemic areas since the 1980's, and this campaign has reduced the infection rate down to 2.9% and the worm burden of those infected to a lower level [1]. Clonorchiasis patients suffer chronically from fatigue, jaundice, abdominal discomfort, and indigestion. *C. sinensis*-infection provokes inflammation, hyperplasia of the biliary epithelium and periductal fibrosis of intrahepatic bile ducts [2]. Furthermore, *C. sinensis* infection can initiate the development of cholangiocarcinoma in biliary epithelial cells in the presence of chronic inflammation and hyperplastic proliferation [3,4]. In fact, it has been shown epidemiologically that clonorchiasis increases the prevalence of cholangiocarcinoma in endemic areas [5]. To prevent cholangiocarcinoma development, early diagnosis and treatment are crucial.

Serodiagnosis is considered to support a diagnosis of clonorchiasis, and to obtain satisfactory serodiagnostic results, the antigenic proteins employed should be sensitive and specific. In fact, the searches conducted to identify better antigenic proteins from excretory-secretory (ES) and somatic proteins propelled biochemical and molecular biological research on *C. sinensis*.

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At the host-parasite interface, *C. sinensis* mechanically stimulates adjacent biliary epithelium and biochemically even stimulates remote biliary epithelium. It is obvious that the ES products of *C. sinensis* contain components that provoke pathologic changes in biliary epithelium, and molecules produced by *C. sinensis* have been shown to stimulate inflammation and the productions of endogenous bioreactive radicals in epithelial cells, and these radicals not only cause DNA damage but inhibit the repair of DNA damage, and thus, promote mutation [3,5]. The molecules responsible and the pathologic progress occurring in and around the biliary tract have long been of research interest. Much research had been conducted to identify the molecules and signal pathway networks involved, and several factors in *C. sinensis* ES products that provoke intracellular signals have been identified [6,7].

Information on the functioning genes can be obtained cost-effectively by collecting and annotating expressed sequence tags during the developmental stages of parasites. In *C. sinensis*, expressed sequence tags were collected from the adults and metacercariae, and annotated and analyzed by referring to the transcriptome and genome information of *Schistosoma mansoni, Schistosoma japonicum,* and *Opisthorchis viverrini* and to genetic databases in the public domain [8-12]. Genetic information on functional genes at the transcriptome level can provide important practical information to proteomic researches and enable comprehensive life phenomena of *C. sinensis* to be elucidated by high throughput microarray analyses.

CHROMOSOMES AND GEOGRAPHICAL ISOLATES

C. sinensis has 2n = 56 chromosomes, where n consists of 8 large and 20 small chromosomes. *C. sinensis* geographical isolates collected in Korea and northeastern China (Liaoning Province) have all demonstrated the same chromosome number, but different karyotypes. Specifically, Korean isolates have 3 metacentric and 1 meta-/submetacentric pairs, whereas Chinese isolates have 2 and 2 pairs, respectively. The 2 isolates have same number, 16 and 8, of submetacentric and subtelomeric pairs [13].

Genetic relationships between geographical isolates have been studied by employing sequence analyses of nuclear ribosomal DNA (18S, internal transcribed spacer 1 and 2; ITS1 and ITS2) and mitochondrial DNA (cytochrome c oxidase subunit 1 [CO-1]). *C. sinensis* Korean isolates collected in Kimhae city were compared with Chinese isolates collected in Shenyang, Liaoning and Nanning, and Guangxi provinces. The geographical isolates were nearly identical in terms of nuclear ribosomal and mitochondrial DNA sequences, and revealed no more than a 1% sequence difference between Korean and Chinese isolates [14-17]. Isozyme electrophoresis had been used to study trematode systematics, and isozymes of the Korean and Chinese isolates show homozygous monomorphic banding patterns. Furthermore, alpha-glycerophosphate dehydrogenase produced a unique band pattern and was found to differentiate geographical isolates of *C. sinensis* [14]. However, in *C. sinensis*, intraspecific genetic variations among geographical isolates in the Sino-Korea region appear minimal.

As compared with *O. viverrini*, *C. sinensis* ITS2 revealed 95% identity and differences at 28 nucleotide points. Furthermore, the mitochondrial CO1 gene of *O. viverrrini* was found to be 96% identical with that of *C. sinensis*. Even a different genus in the same family Opisthorchidae, namely, *O viverrrini* appears to be genetically close to *C. sinensis* [17].

Genome variation

C. sinensis has more than 100 copies of an uncorrupted long-terminal-repeat retrotransposon (*CsRn1*) distributed over its genome. The functional domains of Gag, proteinase, reverse transcriptase, Rnase H, and subdomains of integrase are strongly conserved in *CsRn1*, which has been predicted to be mobile based on structure considerations and from the presence of mRNA transcripts. *CsRn1* belongs to the *Ty3/gypsy*-like long-terminal-repeat transposon family [18]. Multiple copies of the *CsRn1* were clustered into 4 subsets. Sequential transposition of these subsets into the genome was evidenced by the differential sequence divergence and heterogeneous integration patterns of *CsRn1*. Insertions of *CsRn1* appear preferentially at repetitive and agenic chromosomal regions. Furthermore, *CsRn1* was reported to induce variations in the genome that may influence evolution of the *C. sinensis* [19].

EXPRESSED SEQUENCE TAGS

Information on genes expressed in parasites has been collected from diverse taxa, from individual genes, or in massive amounts by the high-throughput sequencing of transcriptomes. In the later case, the mRNAs extracted from an organism are converted into cDNAs to constitute a cDNA library, and sequenced from the 5′-end of each clone to produce expressed sequence tags (ESTs). Functional genes in a parasite can be profiled cost effectively by collecting ESTs under an experimental conditions or a developmental stage.

A total of 3,221 ESTs of *C. sinensis* has been registered in public dbEST databases (http://www.ncbi.nlm.nih.gov/dbEST); 2,802 ESTs from the adults and 415 from the metacercariae [8, 9,20]. In adult *C. sinensis*, the genes abundantly expressed in decreasing order were; groups of metabolic enzymes, regulatory and signal proteins, structure and cytoskeletal proteins, transcription and translation machinery proteins, and proteases and inhibitors. *C. sinensis* adults utilize large amounts of exogenous glucose to produce energy and to provide metabolic intermediates for physiologic processes [21]. As a result, enzymes of the glycolytic pathway are abundantly expressed to drive energy production. Mitochondrial genes expressed at elevated levels also participate in energy production. Furthermore, genes of muscular components, such as limpet homologue, alpha-tubulins, and actin-binding protein, have been identified in second most expressed EST populations; this implies that adult flukes move actively to abrade and feed on biliary epithelia and host blood cells. Cysteine proteases were also found to be expressed at particularly high levels. The proteolytic activities of these proteases help the fluke abrade biliary epithelium and digest engulfed tissue debris and blood cells.

Vitelline precursor protein is abundantly expressed in the adult, which produce a large number of eggs to perpetuate life cycle. The vitelline precursor protein is produced in vitelline glands by yolk cells, which also surrounds germ cells. By stimulation

of the Mehlis' gland, vitelline precursor protein is begun to be secreted and to form the eggshell [22].

In *C. sinensis* metacercariae, the genes abundantly expressed are those of structural and cytoskeletal proteins, energy and other metabolic enzymes, transcription and the translation machinery, the kinases and phosphatases, the DNA scaffold and binding proteins, and the proteases and their inhibitors [9]. The genes expressed higher in metacercariae than in the adult are those of structural and cytoskeletal proteins, kinases and phosphatases, and of the DNA scaffold and binding proteins. Of the annotated ESTs, 26.3% are of structural and cytoskeletal proteins, implying that the metacercariae are in rest phase and maintain minimal metabolism in fish hosts due to the cold environment.

DIFFERENTIALLY EXPRESSED GENES

Gamma-ray treatment of metacercariae prior to infection dose-dependently decreased the recovery rates of adult flukes from experimental animals. However, no chromosome aberrations were found among surviving adult flukes, other than a reduction in chromosome size in some flukes irradiated with 30 Gy [23]. *C. sinensis* metacercariae are relatively resistant to gamma irradiation [24]. In gamma-irradiated *C. sinensis* metacercariae, 19 genes were found to be up-regulated by annealing control primer (ACP)-based PCR, and the up-regulated genes were associated with energy metabolism, protein processing, and DNA repair. It was suggested that the upregulated genes orchestrate DNA and cellular damage repairs induced by gamma-irradiation in metacercariae [25].

Bile is a stimulant to organisms that colonize the mammalian intestine. *C. sinensis* metacercariae excyst in the duodenum, and newly excysted juveniles migrate, driven by bile-chemotaxis to the bile duct and there mature to adults [26,27]. The incubation of *C. sinensis* metacercariae in bile containing media induced the differential expressions of 16 genes, and the corresponding gene products were found to be associated with energy generation or the cell proliferation signal pathway. Bile induces *C. sinensis* metacercariae genes that participate in energy metabolism and modulate the regulatory signals of cell proliferation and of the growth and development of newly excysted juveniles [28].

ANTIOXIDANT ENZYMES

The glutathione *S*-transferases (GSTs) are a family of antioxidant enzymes that catalyze the conjugations of reduced glutathione to electrophilic radicals of a wide variety of substrates. This conjugation activity neutralizes endogenous and exogenous bio-reactive intermediates. Furthermore, GSTs also bind to hydrophobic substrates and transport the GST-substrate complexes to some cellular sites [29]. GSTs have been found ubiquitously in animals from protozoa to vertebrates and are classified as α, μ, π, θ, ζ, σ, and ω classes [30].

Four cytosolic GSTs have been identified in *C. sinensis* with estimated molecular mass of 24.3, 24.7 (2 clones with different peptide sequences) and 25.1 kDa [31-34]. The *C. sinensis* GSTs show enzymatic activity toward 1-chloro-2,4-dinitrobenzene (CDNB). GSTs with molecular masses of 24.3 and 24.7 kDa appear to be homologous in terms of peptide sequence and secondary structure with the 28 kDa GSTs of helminthes and vertebrates. These 3 GSTs were found to be sensitively inhibited by different inhibitors used for the classification. Using these biochemical and enzymatic properties, these 3 GSTs were grouped into the sigma class. The GST of 25.1 kDa revealed molecular and enzymatic properties homologous with the 26 kDa GSTs of invertebrate animals and was allocated to class μ GST [32].

In adult worms, 28 kDa GSTs play the major role in antioxidant activity rather than 26 kDa GST, because the 2 GSTs' molar ratio is 14:1 in the adult [31]. *C. sinensis* 28 and 26 kDa GSTs are localized in the tegument and mesenchymal tissues and in intra-uterine eggs [31,35,36]. Based on their enzymatic activities and localizations in *C. sinensis* adults, they are believed to play a role in the secondary defense system against exogenous and endogenous bioreactive compounds. Whereas other antioxidant enzymes, such as, superoxide dismutase and glutathione peroxidase are abundantly found in the teguments of trematodes and play a role in the primary defense system against exogenous bioreactive compounds and endogenous radicals resulting from hydroperoxidation [37]. The *C. sinensis* 28 and 26 kDa GSTs are localized to the reproductive system, such as, to ovary and sperm and intra-uterine eggs. These antioxidant enzymes could play a defense role against bioreactive species during the reproduction of *C. sinensis* [31-36,38].

Phospholipid hydroperoxide glutathione peroxidases, CsGPx1, CsGPx2, CsGPx3, and CsGPx4, were cloned and localized in the vitellocytes of vitelline glands and in the intrauterine premature eggs of adult *C. sinensis*. CsGPx proteins were co-localized with glutathione in vitellocytes and eggs, whereas thioredoxin was found principally between embryonic cell masses and eggshells [39]. In *S. mansoni*, glutathione peroxidase is pre-

sent at low activity in the tegument of adult flukes, and thioredoxin is secreted from eggs [37]. Glutathione peroxidase, thioredoxins, and peroxiredoxins are crucial for the protection of developing embryos from reactive oxygen radicals derived from endogenous metabolism and from hosts [39,40].

Furthermore, recombinant *C. sinensis* 26 kDa GSTs with or without an oligopeptide of 14 or 48 amino acids at its N-terminus were crystallized and diffracted to 2.3 Å resolution. The crystallization system used for CsGST-peptide fusion proteins could also be applicable to studies of the crystallographic structures of small peptides [41].

PROTEASES

Proteases are ubiquitous enzymes that take part in diverse biological functions in organisms ranging from viruses to man. Recent advances in genomic analysis have revealed that proteases comprise approximately 2% of the total number of proteins in all types of organisms [42,43]. Proteases have been classified into functional groups based on whether they use the hydrolytic mechanisms used by serine, threonine, aspartate, metallo- or cysteine proteases [43]. Furthermore, they function not only as individual enzymes but often in cascades or networks [43]. Genomic and proteomic analysis of several major global helminth parasites have revealed that parasite-derived proteases are key virulence factors [44-52]. The proteases of helminth parasites play numerous indispensable roles in parasite physiology, such as, in protein processing and the turnover of parasite proteins, and in various pathogenic aspects, such as, the facilitation of parasite penetration or invasion into host tissue, the hydrolysis of host proteins for nutrient uptake, and host immune system modulation [46,53-59]. Accordingly, their activities are essential for parasite survival and growth, which suggests that they are attractive targets for vaccines or chemotherapeutic agents [53,60-63]. Therefore, the biochemical and functional characterizations of the proteases of helminthes and their medical applications have been well studied as host issues.

Analyses of the expressed sequence tags (ESTs) of *C. sinensis* have revealed that proteases constitute a large proportion of the protein population of the flukes [8,9], which implies their physiological significance. The cysteine proteases of *C. sinensis* are developmentally regulated and are essential for fluke survival in terms of the contributions they make to biological processes, such as, stage transition, nutrient uptake, and immune evasion [64,65]. Furthermore, the partially purified cysteine proteases from ES products of *C. sinensis* adult worms have been found to have cytotoxic effects on cultured cells [66,67]. In addition, the endogenous cysteine proteases of *C. sinensis* metacercariae appear to be involved in excystation of metacercariae [68].

Several genes encoding cysteine proteases have been isolated from *C. sinensis* [15,67,69-71], and phylogenetic analysis showed that these enzymes are most closely related to the mammalian cathepsin F enzymes [67,71]. It is not yet clear whether *C. sinensis* also expresses and secretes cathepsin L-like enzymes in addition to cathepsin F enzymes. Transcripts encoding cathepsin L-like enzymes are not listed among currently available *C. sinensis* ESTs, although transcripts encoding several other Family C1 proteases, such as, cathepsin B's have been found. The definitive biological functions of these cathepsin F enzymes are not yet clear, but a recent study demonstrated that they participate in nutrient uptake in *C. sinensis*. The biochemical analysis of CsCF-6, a recently identified cathepsin F of *C. sinensis*, suggests that the enzyme is a typical cathepsin F-like enzyme with broad substrate specificity against various human proteins [71]. The enzyme is mainly localized in the intestine of *C. sinensis* and is abundantly identified among ES products [71]. These findings implying that CsCF-6, which is synthesized in the intestinal epithelium of *C. sinensis* and secreted into the intestinal lumen of the parasite, digests various host proteins and might play an important role in nutrient uptake by *C. sinensis*. Proteomic analysis of the ES products of *C. sinensis* adults also found that a large number of cysteine proteases present as major components [72]. The fact that cysteine proteases are secreted into ES products suggests that act as digestive agents in the intestine. A recent study on cathepsin F of *O. viverrini* revealed enzyme in epithelial cells lining the bile ducts of infected animals, which suggested to possibility that it may stimulate biliary epithelium inflammation and proliferation and promote cholangiocarcinogenesis [57]. Hence, the extracorporeal roles of the cysteine proteases of *C. sinensis* warrant further investigation. Furthermore, their involvements in biological roles essential to parasite survival in the host make the helminth cysteine proteases likely targets of novel vaccines [53, 73]. In addition, to the cathepsin F class cysteine proteases currently available *C. sinensis* EST sequences have revealed that the parasite possesses a large number of proteases of different classes or clades.

ENZYMES OF ENERGY METABOLISM

Adult *C. sinensis* dwell in the bile duct, an anaerobic environ-

ment, and therefore, run anaerobic metabolism utilizing large amounts of exogenous glucose as a carbon source for energy metabolism. By utilizing the glycolytic pathway, glucose is converted into energy and provides metabolic intermediates for other physiologic pathways. Phosphoglycerate kinase (CsPGK), a glycolytic enzyme, has been cloned and produced as a enzymatically active recombinant protein in vitro. This enzyme was found to be localized extensively in the muscular tissues of oral and ventral suckers, ovary, testes, and tegument, and in intrauterine eggs [74,75]. Phosphoglycerate mutase, a glycolytic enzyme, of *C. sinensis* was found to catalyze the conversion of 3-phosphoglycerate to 2-phosphoglycerate in the present of cofactor; and its enzymatic activity was found to be inhibited by vanadate [76]. Lactate dehydrogenase (CsLDH) showed no inhibition by high concentration lactate and NAD^{+}, but greatly inhibited by Cu^{+2}, Fe^{+2}, or Zn^{+2}. Furthermore, Gossypol was found to inhibit CsLDH, and thus, was recognized as a potent candidate treatment for *C. sinensis* [77].

Cytosolic and mitochondrial malate dehydrogenases (CscMDH and CsmMDH) of *C. sinensis* share low amino acid sequence homology (22%), but show high MDH enzymatic activity, without lactate dehydrogenase activity or NADPH selectivity. However, these enzymes are differentially inhibited by 4,4′-bisdimethylamino diphenylcarbol. The CscMDH is more stable against heat and acidity than CsmMDH. The malate-aspartate shuttle pathway provides an important mean for the shuttle exchange of substrates and reducing equivalents between cytosol and mitochondria. Furthermore, cMDH plays a pivotal role on the cytosolic side of the malate-aspartate shuttle. mMDH is a key enzyme in the tricarboxylic acid cycle and in the malate-aspartate shuttle. Zheng et al. [78-80] have demonstrated that glycolytic enzymes are essential required for the survival and pathogenesis of *C. sinensis* [78-80].

MEMBRANE PROTEINS

The cytolytic pore-forming peptides are synthesized in the cells of a wide range of animals from protozoa to mammals and are secreted in recognition of their targets. The cytolytic peptides participate in primary defense against infective agents, as they have cytolytic effects on bacteria in food vacuoles and in the environment, and target host cellular components [81]. A pore-forming peptide, clonorin, has been cloned from adult *C. sinensis*, and was found to have 4 amphipathic α-helices and invariably conserved cysteine residues. Clonorin is expressed developmentally in the juvenile and adult stages, and distributes exclusively in the intestinal epithelium of adult flukes. Furthermore, clonorin has hemolytic dose-dependent activity, and is inhibited by specific immune sera. It has been suggested that clonorin could enhance the proteolytic digestion of cellular components in the intestine [82].

In addition, a pore-forming subunit of ATP-sensitive potassium channel (CsKir6.2) cloned from *C. sinensis* adult has 2 transmembrane domains and a GFG-motif in its pore-forming loop, which is a conserved feature of kir channels. Small amounts of the *CsKir6.2* transcript have been detected in the adult stage [83].

ANTIGENIC PROTEINS

Human infections with *C. sinensis* have been confirmed by microscopic findings of eggs in stool samples. Serodiagnostic methods have been devised using antigenic proteins of *C. sinensis* in crude or molecularly defined forms as alternative or complementary diagnostic methods. Using crude antigenic preparations these methods showed higher sensitivities but low specificities. On the other hand, some molecularly defined and recombinant antigenic proteins of *C. sinensis* were found to have markedly improved specificities and limited sensitivities.

Antigenic proteins of *C. sinensis* have been largely found in ES products or purified from soluble extracts of worm lysates. These antigenic proteins are derived from the intestinal epithelium and its contents, namely, the excretory bladder, vitelline follicles, and tegument, as evidenced by immunohistochemical staining using infected or immunized animal sera [84,85]. During early stage infections (< 20 weeks), antigenic molecules were found to be proteins > 34 kDa derived from tegument, testes, or intra-uterine contents, and during the later stage, the major antigenic proteins were found to have molecular weights in the range 29-26 kDa and to be derived from the intestine, excretory, or reproductive organs [86].

Excretory-secretory (ES) antigens

A glycine-rich *C. sinensis* protein (GRCsP) containing 23 repetitions of AQPPKSGDGG was found to be localized in vitelline follicles. Furthermore, recombinant GRCsP protein showed high sensitivity and specificity by ELISA for clonorchiasis [87,88]. Furthermore, a proline-reach antigen (CsRPA) containing 15 GPDAPVPKSG repeats was found to have high sensitivity and specificity against clonorchiasis sera [89].

In addition, a 7 kDa antigenic protein was purified from the

ES products of adult *C. sinensis* and localized to uterine contents and tegumental syncytium. This protein was found to be reactive to clonorchiasis sera but not to paragonimiasis sera [90]. An egg products protein of molecular weight 28 kDa was also found to show high reactivity to clonorchiasis patient sera, but it cross-reacted strongly with the sera of paragonimiasis, opisthorchiasis, and schistosomiasis patients, although it did not react with cysticercosis or sparganosis patient sera [91].

Myoglobin is an abundant protein in ES products, and is localized throughout parenchymal tissues other than those of the reproductive organs of adult *C. sinensis*. In the bile duct, myoglobin may play an oxygen-capturing role and slowly release this oxygen to metabolic pathways. Recombinant myoglobin has been reported to react to 50% of *C. sinensis*-infected rabbit sera and to 25% of clonorchiasis patient sera [92,93], whereas recombinant clonorin showed 100% specificity but low sensitivity for clonorchiasis patient sera. In experimental rabbits, clonorin-specific IgG antibody remarkably increased and remained high at 8 weeks to 1 year after *C. sinensis* infection [94].

Lysophosphatidic acid phosphatase (LPAP) belongs to the acid phosphatase family and hydrolyzes lysophosphatidic acid, a bioactive phospholipid that enhances cell growth, fibroblast chemotaxis, and stimulates neurite retraction. LPAP was identified as an ES-antigen of adult *C. sinensis* and showed higher sensitivity and specificity than crude worm antigen preparations for the serodiagnosis of human clonorchiasis by ELISA [95]. On the other hand, lysophospholipase catalyses the hydrolysis of lysophospholipids to glycerophosphate derivatives and fatty acids, and fatty acid-binding protein plays a role in the intracellular transport of long-chain fatty acids. Lysophospholipase (25.2 kDa) and fatty acid-binding protein (15.2 kDa) cloned from *C. sinensis* adults was found to reactive positively toward clonorchiasis patient sera, but were unsatisfactory as a diagnostic reagent [96,97].

Cysteine proteases of *C. sinensis* are highly antigenic, and thus, their immunodiagnostic values as diagnostic antigens have been investigated [15,67,69,70,98]. The results obtained suggest that the cysteine proteases of *C. sinensis* are reliable serodiagnostic antigens for clonorchiasis [99], but the methodology required for serodiagnosis remains to be determined.

Furthermore, recombinant 26 and 28 kDa glutathione S-transferases have been reported to react with IgG antibodies in patient sera with low sensitivity, but considerations of the highest specificity expected favored further investigations on multi-antigen cocktails [100]. In addition, 2 glutathione S-transferases have been reported to increase *C. sinensis*-specific IgE antibody levels in clonorchiasis patient sera [31,38].

Tegumental proteins

The tegumental syncytium is the outermost surface of human-infecting trematodes, and thus, plays crucial functions at the host-parasite interface. In fact, this surface layer secrets defensive molecules that neutralize host-originated immune and bioreactive radicals. Tegumental proteins are currently the leading vaccine candidates for schistosomiases [101,102]. A tegumental protein of 20.8 kDa (CsTP20.8) has been cloned from adult and metacercariae cDNA libraries and localized to the outer surface membrane of *C. sinensis*. However, recombinant CsTP20.8 protein was considered of limited use for the serodiagnosis of clonorchiasis because it showed moderate sensitivity and high specificity. Oral administration of the CsTP20.8 protein provoked specific IgA production, detected in feces [103,104]. Furthermore, a tegumental protein of 31.8 kDa (CsTP31.8) was immunolocalized to the tegument of adult *C. sinensis*, and has been suggested to be an antigenic protein for the serodiagnosis of clonorchiasis [105].

PERSPECTIVES

C. sinensis thrives in bile juice, which is a biochemically formidable environment. However, bile juice constitutes a favorable environment for *C. sinensis*, and it also plays crucial roles as a growth stimulatory factor and physiological regulator. In the post-genomic era, genetic information on whole genomes and transcitomes provides an essential and fundamental platform, which facilitates approaches based on functional genomics and bioinformatics and provides an overview of the complexities of life phenomena, physico-metabolism, and responses to external stimuli. Bile-chemotaxis attracts *C. sinensis* juveniles into bile ducts, and bile components stimulate the activities and growths of these juvenile flukes. Accordingly, we consider that the biological significance of bile to *C. sinensis* deserves study in depth with respect; its influence on the biological aspects of cellular signaling networks, the genes involved, and the neurologic circuits that empower the chemotactic migration of juvenile flukes. Single antigenic proteins have been evaluated, but have been found to have low sensitivity, though high specificity, for the serodiagnosis of *C. sinensis* infections. Multiple antigen cocktails involving optimizations of specific antigenic epitopes offer an alternative approach. However, to achieve this

target, a large number of proteins of high specificity must be identified using forward and reverse genetic approaches and molecular biological analyses. As *C. sinensis* is identified to be a biological agent for cholagiocarcinoma, researches should be pursued on carcinogenic material derived from *C. sinensis* and on molecular networks and genetic regulation of cholangiocarcinogenesis in the biliary system.

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